Effects of Monoclonal Anti–c-Kit Antibody (ACK2) on Melanocytes in Newborn Mice

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Previous studies indicate that c-Kit is required for postnatal melanocyte development. To understand the precise mechanisms of c-Kit dependence, we studied melanocyte development in newborn C57BL/6 mice by means of peritoneal injection of a monoclonal anti–c-Kit antibody (ACK2), which blocks c-Kit functions. The mice were injected once or more with ACK2 at various intervals after birth. In experiment 1, skin samples were examined on day 10 post-partum and in experiment 2 they were examined daily until day 10 post-partum. We studied melanocytes in the hair follicles, epidermis, and dermis by light and electron microscopy with dopa reactions and immunohistochemistry. Epidermal melanocytes in untreated mice were dopa negative and c-Kit positive on day 0 post-partum but became dopa positive soon thereafter. In ACK2-treated mice, the earlier the mice received ACK2 injections after birth, the fewer melanocytes they had, not only in the epidermis, but also in follicles. In these mice, melanocytes that had undergone apoptosis in the dermis and the follicles were detected ultrastructurally. Some appeared to have produced tyrosinase, because they had dopa-positive melanosomes. These results suggest that melanocytes in newborn mice are c-Kit dependent and undergo apoptosis when c-Kit receptors are blocked by ACK2 in the early days after birth. During this c-Kit–dependent period, melanocytes differentiate from dopa negative to positive and migrate from the epidermis to hair follicles. ***Key words: melanocyte development/coat color alteration/apoptosis. J Invest Dermatol 105:322–328, 1995***

Melanocytes are neural crest-derived cells that synthesize melanin pigments by tyrosinase activity. Fully differentiated active melanocytes are found mainly in the hair bulbs of adult mouse skin. In mouse embryos, melanoblasts, precursors of melanocytes, migrate lateroventrally to the dermis, where they invade the epidermis between days 11 and 12 of gestation [1,2]. Epidermal melanoblasts begin to differentiate into active melanocytes on day 16 of gestation [3] and both melanoblasts and active melanocytes are located in the epidermis at birth. These melanocytes increase dramatically in number after birth [4–6] and migrate into the hair bulbs [3,7], giving rise to melanized hair.

c-kit is a gene encoding a tyrosine kinase receptor and has been mapped to the dominant white spotting (W) locus. In addition, cDNA of the ligand for murine c-Kit (*c-kit* protein), stem-cell factor (SCF), has been cloned [8–10] and mapped to the Steel (Sl) locus [8,9,11], whose phenotypes are basically identical to W [12]. Phenotype analysis of W and Sl mice has demonstrated the involvement of c-Kit/SCF in melanocyte development at the embryonal stage, although previous studies have not specified which stage of melanocyte development functionally requires c-Kit and/or SCF for further differentiation. To address this question, Nishikawa *et al* used a monoclonal anti–c-Kit antibody (ACK2) [13], an antagonistic blocker of c-Kit function, to interfere with melanocyte development during embryonic and postnatal life. The following conclusions were drawn by examining the changes in coat color of the mice: 1) functional c-Kit is required during mid-gestation for a limited period when melanocytes invade the epidermis from the dermis and 2) c-Kit is required for postnatal melanocyte activation, which occurs concomitantly with neonatal development of the first hair and hair cycle, although the precise mechanisms by which ACK2 affects melanocytes remain unknown.

In this study we investigated these mechanisms using wild black neonatal mouse skin with and without ACK2 injections and by performing light and electron microscopic dopa reactions, immunohistochemical staining with ACK2, and ordinary light and electron microscopy. This study was designed to understand a) c-Kit expression of melanocytes in neonatal mice, b) which melanocyte development stages are affected by ACK2, and c) the mechanisms by which ACK2 affects melanocytes.

MATERIALS AND METHODS

Mice C57BL/6 newborn mice were born to pregnant mice purchased from Japan SLC, Inc. The newborn mice were raised by their mothers.

ACK2 We used a monoclonal ACK2 [13,14], which is not only a marker of c-Kit positive cells but also a blocker of c-Kit function *in vivo*. The antibody, created by Nishikawa *et al*, is Rat IgG2b, and it can pass through the placenta.

ACK2 Administration ACK2 was administered to the peritoneal cavity of C57BL/6 newborn mice in single doses of 50 μg. The days of

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Abbreviations: ACK2, anti–c-Kit antibody; SCF, stem-cell factor; W, white spotting.

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administration were varied depending upon the experimental objectives. Experiment 1 was conducted to investigate changes in coat color and changes in melanocytes induced by ACK2. Experiment 2 was conducted to investigate how ACK2 affects melanocytes. **Tables I and II**, respectively, describe the administration and frequency used in experiments 1 and 2. The mice, including a control group that was not injected with ACK2, were anesthetized with ether. Coat color was observed and photographed, and skin samples were taken from the dorsal and abdominal areas. These skin samples were then processed for ordinary light and electron microscopic examinations, light and electron microscopic dopa reactions, and immunohistochemistry. Fresh skin samples were fixed with 20% formalin and embedded in paraffin. Sections were cut and stained with hematoxylin-eosin and toluidine blue and observed under a light microscope. Skin samples were embedded in an O.C.T. compound (Chiba Medical Co., Japan), frozen, and cut at 5 μm. Sections were placed on glass slides coated with poly-L-lysine, fixed for 1 h with 10% formalin, and immersed in 0.1% DL dopa (Sigma Chemical Co., St. Louis, MO) at 37°C for 8 h. Thereafter, the slides were stained with Kernechtrot nuclear fast red (Merck, Germany).

To observe the dopa reactions, dopa-positive melanocytes with clear dendrites were counted per unit area (mm^2) of the epidermis. In hair-bulb areas, hair bulbs with clearly visible papillae were selected. Because many melanocytes were densely packed in the hair bulbs thus making them difficult to count individually, we divided the number of dopa-positive hair bulbs by the total (dopa-positive hair bulbs/total number of hair bulbs).

Immunohistochemistry Frozen sections 5 μm thick were placed on glass slides and fixed in 100% acetone at 5°C for 10 min. The primary antibody, ACK2, was diluted a hundredfold with bovine serum to a concentration of 10 μg/ml and stained using Histstain-SP KIT (Zymed Laboratories, CA) to identity the cells upon the surfaces of which c-Kit appeared. Positive melanocytes in which the nuclei were clearly visible were counted per unit area (mm^2) of the epidermis.

Because all c-Kit–positive melanocytes could not be counted in the hair bulbs due to the large number of melanin pigment granules, we calculated a figure for c-Kit hair bulbs by dividing the number of c-Kit (ACK2)–positive hair bulbs by the total (c-Kit–positive hair bulbs/total number of hair bulbs). Only those hair bulbs in which the hair papillae were clearly visible were selected.

Electron Microscopy Skin samples were cut in approximately 3 × 3 mm sections and double-fixed in 2% glutaraldehyde and 1% osmium tetroxide. After routine dehydration and embedding, they were cut into ultrathin sections, double-stained with uranyl acetate and lead citrate, and observed under an electron microscope (JEM-1200EX). For electron microscopic dopa reaction, skin samples were thinly sliced into 3 × 3 mm sections fixed in 2% glutaraldehyde for 15 min, and immersed in 0.1% DL dopa for 5 h at 37°C. After washing of the samples with phosphate-buffered saline, they were fixed with 2% glutaraldehyde for 24 h and 1% osmium textroxide for 1 h and routinely precessed for electron microscopic observation.

Statistical Analysis t tests were used for statistical analysis.

Table I. Dopa-Positive Hair Bulbs/Total Hair Bulbs on Day 10 Post-Partum in Mice Given ACK2 (Experiment 1)[a]

Group	Days Post-Partum of ACK2 Injection	Number of Mice	Dopa Positive Hair Bulbs/ Total Hair Bulbs[b]
A	0, 2, 4, 6, 8	2	0.17 ± 0.21
B	0	3	0.54 ± 0.16
C	2	3	0.72 ± 0.06
D	4	3	0.85 ± 0.04
E	6	3	0.92 ± 0.02
F	8 and 9	2	0.99 ± 0.02
G	None (Control)	5	1.00 ± 0.00

[a] ACK2 was administered to seven groups according to the following schedule: group A, a total of five times on days 0, 2, 4, 6, and 8 post-partum; group B, day 0 post-partum only; group C, day 2 post-partum only; group D, day 4 post-partum only; group E, day 6 post-partum only; group F, on consecutive days 8 and 9 post-partum; and group G, the control group that was not injected with ACK2.

[b] Data are presented as mean ± SD.

RESULTS

Coat Color and Number of Dopa-Positive Hair Bulbs Change with ACK2 Administration Ten days after birth, coat color was examined for each group in experiment 1 **(Table I)** with the naked eye. Coat color changed most (became whitest) in group A, followed by groups B, C, D, E, F, and G in that order. Thus, the earlier ACK2 was administered after birth, the more conspicuous coat whitening became. Under identical administration conditions, coat colors become whiter on the abdominal side than on the dorsal side **(Fig 1*a*)**.

In each group, the number of hair bulbs with dopa-positive melanocytes was counted for comparison. In group A mice, in which coat color appeared white to the naked eye, only a few dopa-positive hair bulbs were found microscopically, and the number of dopa-positive hair bulbs/total number of hair bulbs was

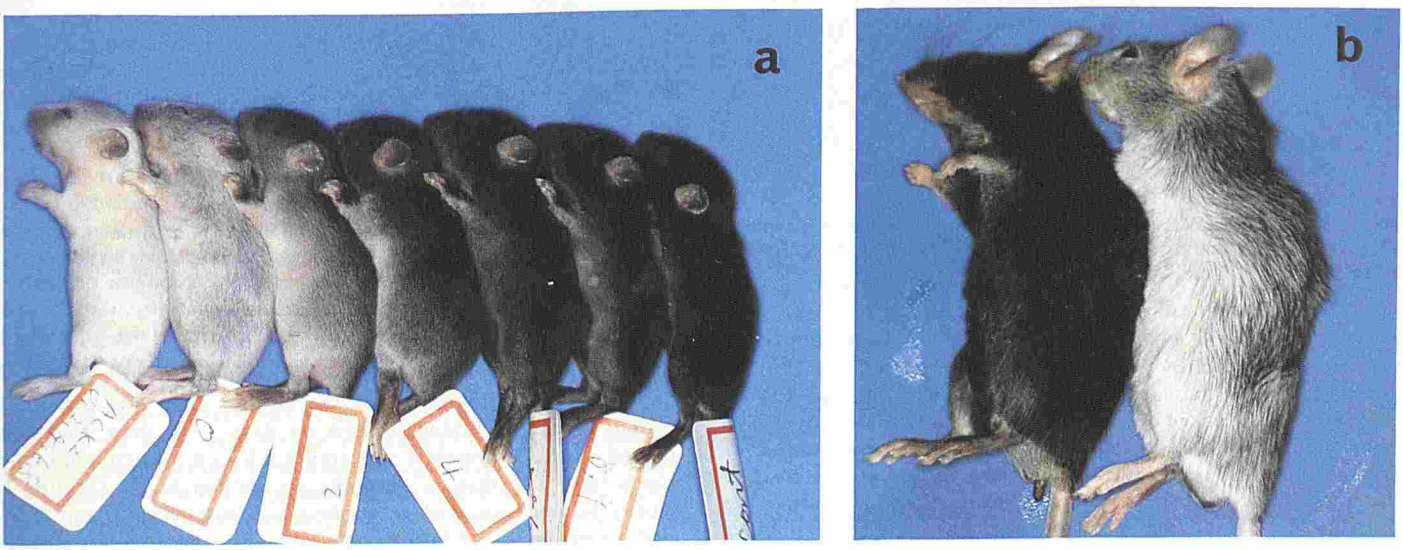

Figure 1. Early ACK2 administration whitens coat color. *a*) Mice on day 10 post-partum. The first mouse on the left was intra-peritoneally injected with 50 μg ACK2 on days 0, 2, 4, 6, and 8 post-partum. The second mouse was injected on day 0 post-partum. The third mouse was injected on day 2 post-partum. The fourth mouse was injected with ACK2 once on day 4 post-partum. The fifth mouse was injected on day 6 post-partum. The sixth mouse was injected on days 8 and 9 post-partum. The last was the control, which was not injected with ACK2. *b*) Mice on day 72 post-partum. The mouse on the left was a control. The mouse on the right was injected with ACK2 on days 0, 1, and 2 post-partum.

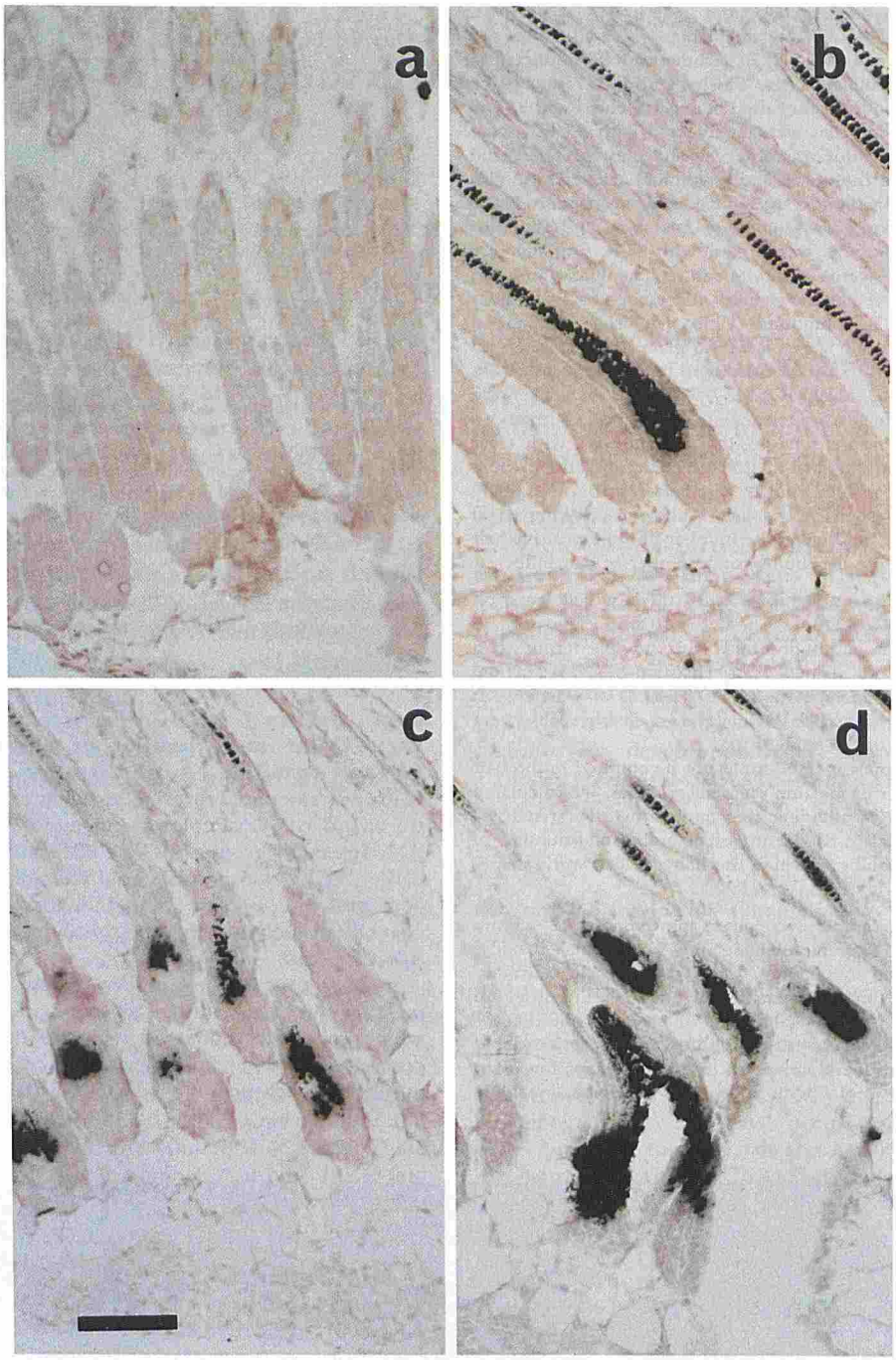

Figure 2. ACK2 administration reduces the number of dopa-positive hair bulbs. The skin samples were taken on day 10 post-partum from mice injected with ACK2 on days 0, 2, 4, 6, and 8 post-partum (*a*); ACK2 injection on day 0 post-partum (*b*); ACK2 injection on day 4 post-partum (*c*); and ACK2 injections on days 8 and 9 post-partum (*d*). Using those samples, the dopa reaction was performed as described in *Materials and Methods*. *Bars*, 100 μm.

the lowest, followed by groups B, C, D, E, F, and G, in that order **(Table I, Fig 2)**. These results were the same as the changes in coat color seen by the naked eye. The earlier and more frequently ACK2 was administered, the greater was the decrease in the number of dopa-positive hair bulbs. At the same time, there was a decrease in the intensity of the hair bulb dopa reaction **(Fig 2)**. Because the intensity of hair bulb dopa reaction varies according to the number of dopa-positive melanocytes, the decrease in these dopa reactions corresponded to a decrease in the number of dopa-positive melanocytes in the hair bulbs.

Skin and Coat Color Successively Change in Mice with or without ACK2 Administration Skin color of the control mice **(Table II**, group J) that were not injected with ACK2 at day 0 appeared pinkish-white and hairless. The skin gradually turned black, becoming darkest on days 4–6, then gradually turned white, although it appeared black as it was covered by a black coat. The skin color of the mice given ACK2 (groups H and I) did not turn as black on days 4–6 post-partum, and some white hairs appeared in the coats as their coats grew. On day 10 post-partum coat color ranged from almost all white to gray. As the mice grew, their coats became darker but were still gray by days 30, 72 **(Fig 1*b*)**, and 389 post-partum, whereas their abdominal areas in particular remained almost completely white. These results demonstrate that ACK2 has a strong effect on

Table II. Mice Examined from 0 to 10 Days Post-Partum After ACK2 Injections (Experiment 2)[a]

Group	Days Post-Partum of ACK2 Injection	Days Post-Partum of Biopsy	Dorsal Skin (n)	Abdominal Skin (n)
H	0 (n = 30)	0	5	1
		1	4	1
		2	2	1
		4	3	1
		6	2	1
		8	3	1
		10	4	1
I	0, 1, 2 (n = 16)	2	3	
		3	2	1
		4	2	1
		6	2	
		8	2	
		10	2	
		30	1	
J	None (control group) (n = 47)	0	5	1
		1	3	1
		2	4	1
		3	3	1
		4	5	1
		5	4	0
		6	6	1
		8	4	1
		10	5	1

[a] Comparisons were made among three groups. In group H ACK2 was administered only on day 0 post-partum. The mice were killed and skin samples were taken on days 0, 1, 2, 4, 6, 8, and 10 post-partum. In group I ACK2 was administered three times on consecutive days 0, 1, and 2 post-partum. The mice were killed and skin samples taken on days 2, 3, 4, 6, 8, 10, and 30 post-partum. In group J ACK2 was not administered. The mice were killed and skin samples were taken on days 0, 1, 2, 3, 4, 5, 6, 8, and 10 post-partum.

melanocytes, particularly in the abdominal area, and that recovery is very slow.

ACK2 Dramatically Affects Epidermal Melanocytes in the Early Post-Natal Stage In the control mice (**Table II**, group J), just after birth, an immunohistochemical examination showed that the number of c-Kit–positive epidermal melanocytes was 17.0/mm^2 in mice on day 0 post-partum. On day 1, there were 29.3 melanocytes/mm^2, reaching a peak on day 4 post-partum at 46.2/mm^2 followed by a gradual decrease to 0.0/mm^2 on day 10 post-partum (**Figs 3*a* and 4*a***). Dopa reaction, which indicates melanocytes containing tyrosinase, showed that the number of melanocytes did not change significantly on days 0 and 1 post-partum, being 5.0/mm^2 and 6.6/mm^2, respectively. From day 2 post-partum, however, the number increased sharply, reaching a peak of 68.2/mm^2 on day 4 post-partum and decreasing to 0.0/mm^2 on day 10 post-partum (**Fig 4*a***). These results indicate that dopa-negative immature melanocytes in the early postnatal stage are c-Kit positive and that epidermal melanocytes disappeared on day 10 in the control mice.

The number of dopa-positive melanocytes for the mice in group H, which were injected with ACK2 on day 0 post-partum only, was 4.9/mm^2 on day 1 post-partum and did not increase any further. On day 2 post-partum the number decreased to 3.0/mm^2 and after day 4 post-partum fell to almost 0.0/mm^2 (**Fig 4*a***). These results were similar to the results in group I, in which the mice were treated with ACK2 on days 0, 1, and 2 post-partum (**Fig 4*a***). The number of dopa-positive melanocytes in the groups given ACK2 decreased to nearly 0.0/mm^2, unlike the control group, in which there was an increase. We deduced from these epidermal melanocyte examinations that the melanocytes within the epidermis were killed by blocking of c-Kit with ACK2.

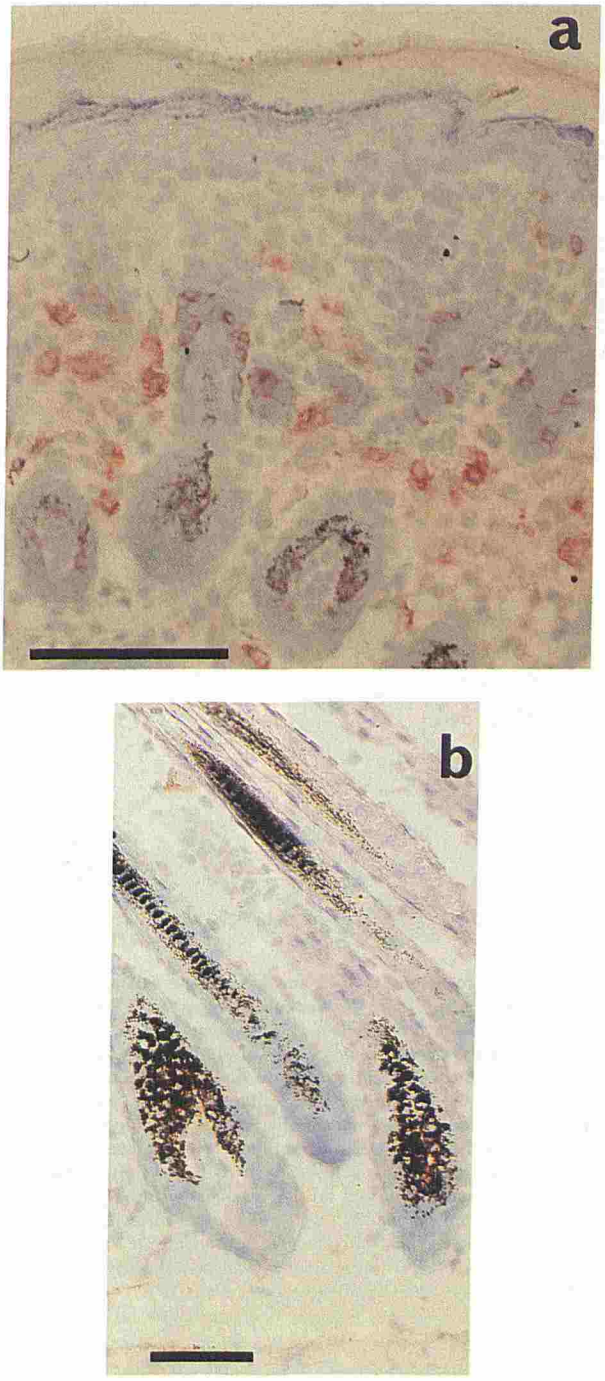

Figure 3. c-Kit–positive melanocytes in control mice appear in the epidermis and in the upper part of hair follicles on day 1 post-partum but they are only found in the hair bulbs on day 10 post-partum. Skin samples were taken from control mice on days 1 (*a*) and 10 (*b*) post-partum. Using ACK2 as a primary antibody, immunohistochemical staining was performed as described in *Materials and Methods*. c-Kit–positive cells in the dermis (*a*) were most likely mast cells because they showed metachromasia from the toluidine blue stain (not shown). *Bars*, 100 μm.

Melanocytes in Hair Follicles Are Affected by ACK2 During Hair Follicle Development In the control group, a large number of c-Kit–positive melanocytes were detected in the upper part of the hair follicle only from days 0 through 6 post-partum (**Fig 3*a,b***), although they were not counted. c-Kit–positive cells disappeared from epidermis (**Fig 4*a***) and the upper part of the hair follicle on day 10 post-partum, and they were only detected in hair

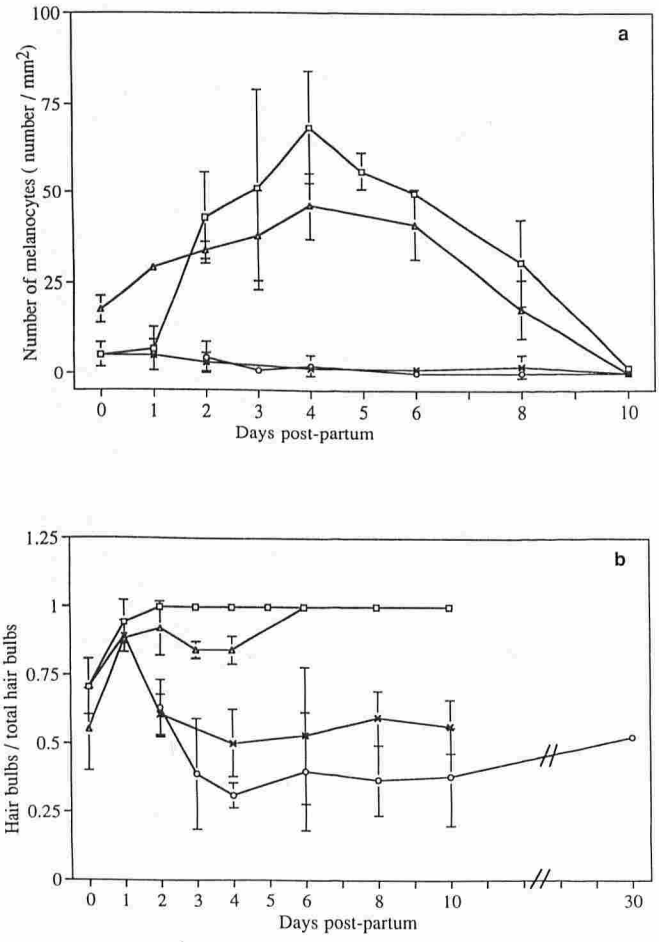

Figure 4. The number of dopa- and c-Kit–positive melanocytes in the epidermis and hair bulbs decrease upon ACK2 administration. *a*) Dopa- and c-Kit–positive melanocytes were counted in the epidermis and the number per unit area (mm^2) was determined on days 0, 2, 4, 5, 6, 8, and 10 post-partum in the control group and the mice injected with ACK2. *b*) The number of dopa- and c-Kit–positive hair bulbs were counted and respectively divided by the total number of hair bulbs in the control group and ACK2-injected mice. All the samples were obtained from the dorsal skin. The results represent mean ± SD of two to six mice **(Table II)**, but only one mouse was examined on day 30 post-partum. (*b*). Dopa-positive melanocytes (*a*) and hair bulbs (*b*) in the control mice (□), in group mice given ACK2 on day 0 post-partum (×), and in group I mice given ACK2 injections on days 0, 1, 2 post-partum (○). c-Kit–positive melanocytes (*a*) and hair bulbs (*b*) in the control (△).

bulbs **(Figs 3*b* and 4*b*)**. Therefore, we speculated that these melanocytes may migrate from the epidermis to the hair bulb during hair follicle development. Melanocytes that had developed somewhat also expressed c-Kit, because those that carried melanin granules were also c-Kit–positive **(Fig 3*b*)**. There were almost no c-Kit–positive hair bulbs found in the groups in which ACK2 was administered during the early period. However, in these groups, c-Kit receptor may already have been bound to ACK2 and was not easily stained by the immunohistochemical method in which the primary antibody was ACK2. Even if the receptors were stained, they would have been difficult to identify due to the melanin granules. Therefore, c-Kit–positive cells were not counted in the immunohistochemical samples from the mice that had been given ACK2. The ratio of the number of dopa-positive hair bulbs to the total number of hair bulbs in the control group was 0.70 on day 0 post-partum, and it increased to 1.00 on day 2 post-partum, indicating that all hair bulbs became positive on day 2 post-partum. This number did not change any further **(Fig 4*b*)**. As the hair follicles grew, the intensity of the hair bulbs' dopa reaction increased. In group H mice, which were injected with ACK2 on day 0 post-partum only, the number of dopa-positive hair bulbs on day 1 post-partum was 0.89, with no significant difference from the control group at 0.94. From day 2 post-partum, however, the number significantly decreased to 0.60 compared with the control group ($p < 0.05$), and it reached the lowest level on day 4 post-partum at 0.50. By day 10 the number had insignificantly increased to 0.56 **(Fig 4*b*)**.

In group I mice, which were injected with ACK2 on days 0, 1, and 2 post-partum, the ratio of the number of dopa-positive hair bulbs to the total number of hair bulbs was lowest on day 4 post-partum at 0.31, and it significantly decreased ($p < 0.05$) compared with the ratio on day 0 post-partum at 0.70 in the control group. There were no significant differences among the ratios on days 6, 8, and 10 post-partum. On day 30 post-partum, about half the melanocytes recovered **(Fig 4*b*)**.

ACK2 Induces Apoptosis in Melanocytes with Dopa-Positive Melanosomes The electron microscopic dopa reaction showed that cells with condensed nuclei and cytoplasm, which were phagocytized by surrounding keratinocytes **(Fig 5*a*)**, were present in hair bulbs of the skin samples taken on day 2 post-partum from the dorsal area of mice given ACK2 on consecutive days 0 and 1 post-partum. Because these cells contained dopa-positive melanosomes **(Fig 5*a*)**, they were judged to be melanocytes **(Fig 5*a*)**. Cells with relatively intact cell membranes and with nuclear chromatin condensed into half-moon shapes were found in the dermis **(Fig 5*b*)** on day 3 post-partum from the abdominal area of the mice injected with ACK2 on day 0 post-partum. In the same samples, cells with condensed nuclei and dopa-positive melanosomes were phagocytized by macrophage-like cells **(Fig 5*c*)**. These electron microscopic findings were the same as those in cells undergoing apoptosis. Although the number of apoptotic melanocytes was very small, more were found in the abdominal than in the dorsal area. No apoptotic melanocytes were found in the control mice that were not injected. There was a large number of hair bulbs without melanocytes in the skin samples taken on day 10 post-partum from the group injected with ACK2 on day 0 post-partum only **(Fig 5*d*)** and from the group with injections on alternating days 0, 2, 4, 6, 8, and 10 post-partum. In the control group without ACK2 injection, melanocytes with a large number of melanosomes were found in hair follicles **(Fig 5*e*)**. Therefore, it is estimated that ACK2 kills melanocytes by mediating apoptosis in them.

No Inflammatory Response in Mouse Skin Injected with ACK2 All the skin samples in the above experiments with hematoxylin-eosin staining showed no significant differences except for changes in melanocytes and the amount of melanin between the groups of mice injected with and without ACK2. Melanocytes disappeared without leaving any histologic findings of inflammation.

DISCUSSION

Nishikawa *et al* [13] have observed newborn C57BL/6 mice injected with ACK2, a monoclonal anti–c-Kit antibody, and reported that coat color was dramatically diluted by ACK2 injections in the early days after birth [13]. These results suggested that melanocytes in the hair follicle development stage are affected by ACK2, although it is not clear at which development stage or how melanocytes were affected, because the observations were only visual. Therefore, we investigated these issues by means of visual inspection as well as light and electron microscopy including dopa reactions and immunohistochemistry using skin samples of C57BL/6 mice injected with ACK2.

Our visual inspection indicated that the earlier ACK2 was administered after birth the more conspicuous coat-whitening became **(Fig 1*a*)**. Our study also showed that this whitening was more noticeable in the abdominal areas **(Fig 1*a*)**. Moreover, later observations of the mice injected with ACK2 on days 0, 1, and 2 post-partum, revealed that coat color in the dorsal areas had

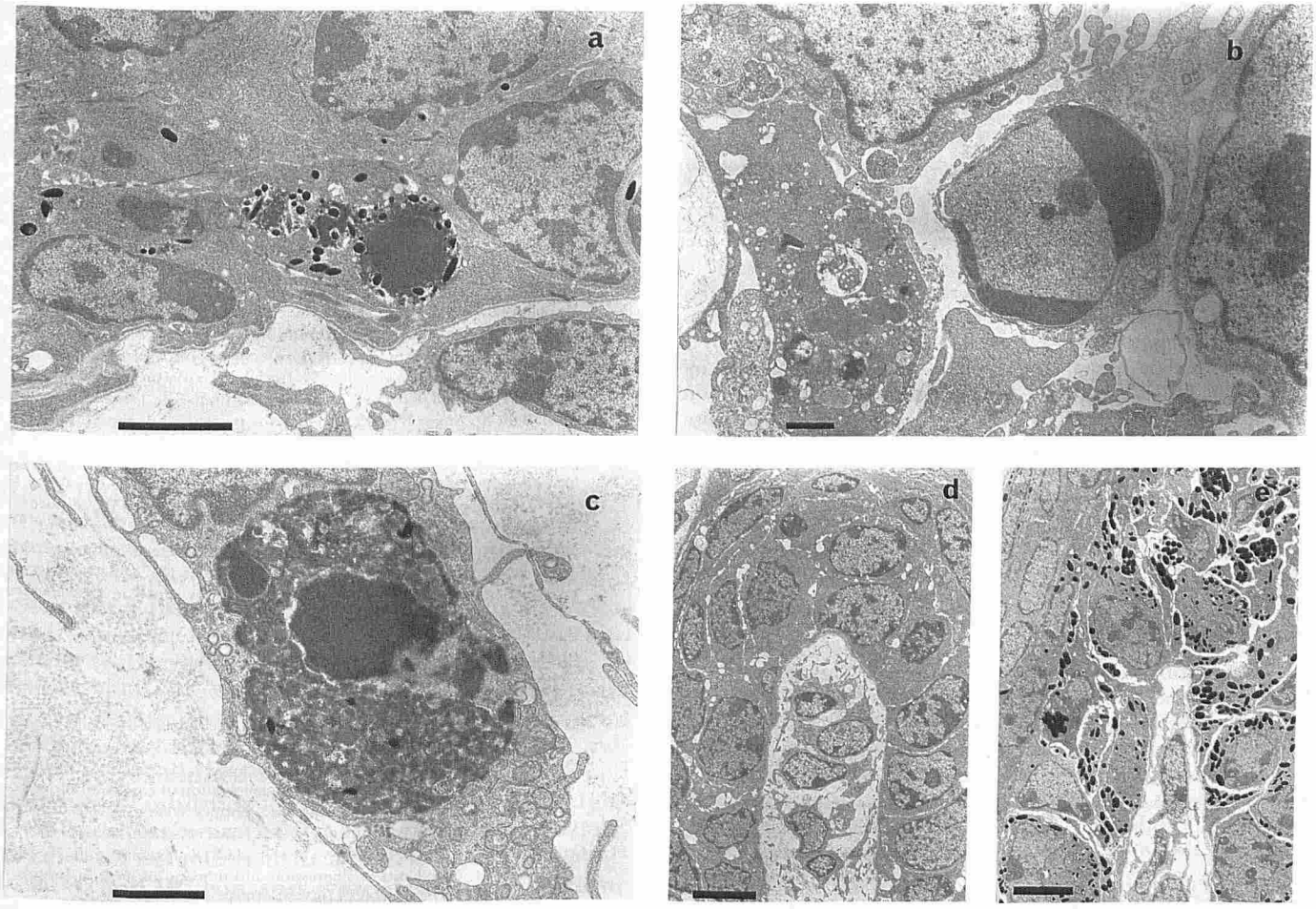

Figure 5. ACK2 kills melanocytes by mediating apoptosis. Skin samples were obtained on day 2 post-partum from the dorsal skin of mouse with ACK2 injection on days 0 and 1 post-partum (*a, bar,* 5 μm), on day 3 post-partum from the abdominal skin of the mouse with ACK2 injection on day 0 post-partum (*b, bar,* 1 μm; *c, bar,* 1 μm), and on day 10 post-partum from the dorsal skin of mice with (*d, bar,* 5 μm) and without (*e, bar,* 5 μm) ACK2 on day 0 post-partum. The electron microscopic dopa was performed as described in *Materials and Methods*. Apoptotic melanocytes are seen in *a, b,* and *c*. No normal melanocytes are seen in *d*, whereas there are in *e* (control).

returned to gray, but the abdominal areas remained white even on days 72 and 389 post-partum **(Fig 1*b*)**. From these findings we deduced that melanocytes die and that not enough survive to completely restore coat color.

We could not precisely explain why the more conspicuous coat-whitening developed in the abdominal area. One possibility is that neonatal melanocytes are more immature and more sensitive to ACK2 in the abdominal area, which is most distant from the neural crest, so melanoblasts arrive there later than in other areas during embryonic life. This is somewhat different from mice with the W/+ genotype and human piebaldism, which are caused by a heterozygous deletion of the c-Kit gene [15]. This deletion results in melanoblasts' producing half of the normal number of receptors (c-Kit), impairing migration, proliferation, and colonization of melanoblasts during embryonic life at sites most distant from the neural crest [15].

To study changes in melanocytes affected by ACK2, it is necessary to know the exact status of normal melanocytes in the neonatal period. The study of the mouse epidermis in the control group mice showed that melanocytes became c-Kit positive before turning dopa positive. This indicated that c-Kit is a good marker for dopa-negative immature melanocytes in the epidermis where mast cells, which are c-Kit positive, do not exist. However, c-Kit positive does not mean immature, because tyrosinase-producing melanocytes that undergo some differentiation and contain melanin granules also express c-Kit. An examination of the upper part of the hair follicles revealed a large number of c-Kit–positive melanocytes on day 1 post-partum, which gradually decreased to zero on day 10 post-partum. The number of c-Kit–positive cells in the hair bulbs, however, increased, and almost all of them became c-Kit positive on day 6 post-partum **(Fig 4*b*)**. Therefore, we estimated that melanocytes reach the hair bulbs through the upper part of the follicle and become c-Kit positive during hair follicle development. On day 10 post-partum, there was a marked decrease in the number of dopa-positive melanocytes in the hair bulbs of mice given ACK2 during the early postnatal period. This number decreased as the frequency of administration increased. Mice that appeared white, however, also had dopa-positive melanocytes remaining in hair bulbs, indicating that differentiated dopa-positive melanocytes are not easily killed by ACK2. Furthermore, when ACK2 was administered after day 6 post-partum, the number of dopa-positive melanocytes did not significantly decrease even though there was a large number of c-Kit–positive cells **(Table I)**. These findings showed that differentiated melanocytes are not c-Kit dependent and are not killed by ACK2, even when they express c-Kit receptors.

To learn more about how ACK2 affects melanocytes, ACK2 was administered on day 0 post-partum and days 0, 1, and 2 post-partum. The test and control groups were examined daily (experiment 2). In the control group mice, epidermal dopa-positive cells increased significantly from day 2 post-partum and peaked on day 4 post-partum, whereas in the test groups the effects of ACK2 were most powerful when it was administered on day 0 post-partum; no dopa-positive melanocytes were found in the epidermis. There

were already a large number of c-Kit–positive immature melanocytes in the control group mice on day 0 post-partum, but most of them were dopa negative **(Fig 4*a*)**, indicating that most of the melanocytes killed by blocking c-Kit with ACK2 just after birth were dopa negative and c-Kit positive.

Light microscopic observations of all the skin samples stained with hematoxylin-eosin revealed that excluding the melanocyte abnormalities there were no abnormal structures or inflammation in the epidermis, dermis, or hair follicles, indicating that ACK2 selectively killed melanocytes. However, the question of the mechanism remains unresolved.

Two explanations can be considered, because ACK2 is an antibody (IgG2b) that can block c-Kit functions. One is that antibody-mediated cytotoxic reactions cause cell death. However, this can be ruled out because mature melanocytes are not killed by ACK2 even though they are c-Kit positive and no inflammation was histologically visible. The other explanation is that because ACK2 blocked the c-Kit receptors, SCF, which is a c-Kit ligand, could not act upon melanocytes, thus causing cell death.

Apoptosis is a distinct type of cell death that differs fundamentally from degenerative death or necrosis in its nature and biologic significance [16]. Definitive morphologic changes in apoptosis have been detected by electron microscopy [16]. In this study, dopa electron microscopy revealed apoptosis in some hair bulbs and in the dermis in mice given ACK2 during the early postnatal period **(Fig 5*a,b,c*)**. We concluded that melanocytes became apoptotic after blocking of c-Kit with ACK2, because no signs of apoptosis were seen in the control group mice and most apoptotic cells contained tyrosinase-positive melanosomes. Because light-microscopic observation did not reveal any inflammation and only melanocytes were selectively destroyed, it is possible that melanocytes underwent apoptosis. However, because the numbers of apoptotic cells in the skin samples were very small, we could not demonstrate the DNA fragmentation that is characteristic of apoptosis. The existence of apoptotic melanocytes with dopa-positive melanosomes indicated that partially developed melanocytes are also c-Kit dependent.

Membrane-bound SCF is necessary for the survival of melanocytes in the mouse embryos [17], but there are no reports describing apoptosis due to administering ACK2 to melanocytes or SCF exclusion. Iemura *et al* [18], however, have reported that mouse mast cells, which have c-Kit receptors and in which differentiation is related to SCF/c-Kit, undergo apoptosis when SCF exposure is stopped *in vivo* and *in vitro*. We suspect that apoptosis occurs because SCF cannot act on melanocytes via the c-Kit receptors, which are blocked by ACK2.

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